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International application number: PCT/US05/000843

International filing date: 10 January 2005 (10.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/535,360
Filing date: 09 January 2004 (09.01.2004)

Date of receipt at the International Bureau: 18 February 2005 (18.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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APPLICATION NUMBER: 60/535,360

FILING DATE: *January 09, 2004*

RELATED PCT APPLICATION NUMBER: PCT/US05/00843



Certified by

Don W. Dudas

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c).

Docket Number		21101.0054U1		Type a Plus Sign (+) inside this box		+																					
INVENTOR(s)																											
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)																								
Bock	Susan	C.	2027 East 900 South, Salt Lake City, Utah 84108																								
TITLE OF INVENTION (500 characters max)																											
METHODS OF USING HIGH AFFINITY ATHI VARIANTS UNDER HIGH WALL SHEAR RATE CONDITIONS																											
CORRESPONDENCE ADDRESS																											
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031431 U.S. PTO
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METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (Check One)

- ☒ Applicant claims small entity status. See 37 CFR § 1.27.
- ☐ A Credit Card Payment Form PTO-2038 is enclosed to cover the filing fees.
- ☐ A check or money order is enclosed to cover the filing fees.
- ☒ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 501977.
- ☒ The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 14-0629.

FILING FEE AMOUNT

\$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- ☐ No.
- ☒ Yes. The name of the U.S. Government agency and the Government contract number are:

Respectfully submitted

Signature

Date

Typed or Printed Name:

David E. Huizenga, Ph.D.

Registration No.

49,026

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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David E. Huizenga

Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)	
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Bock)	Art Unit: Unassigned
)	
Application No. Unassigned)	Examiner: Unassigned
)	
Filing Date: Concurrently)	Confirmation No. Unassigned
)	
FOR: METHODS OF USING HIGH AFFINITY)	
ATIII VARIANTS UNDER HIGH WALL)	
SHEAR RATE CONDITIONS)	

AUTHORIZATION TO TREAT REPLY REQUIRING EXTENSION OF TIME
AS INCORPORATING PETITION FOR EXTENSION OF TIME

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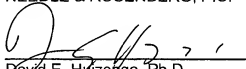
Pursuant to 37 C.F.R. § 1.136(a)(3), the Commissioner is hereby requested and authorized to treat any concurrent or future reply in the above-identified application, requiring a petition for an extension of time for its timely submission, as incorporating a petition for extension of time for the appropriate length of time.

ATTORNEY DOCKET NO. 21101.0054U1
PATENT

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Respectfully submitted,

NEEDLE & ROSENBERG, P.C.



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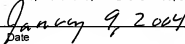
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Express Mail No. EL 992018720US
Attorney Docket No. 21101.0054U1
UTILITY PATENT - PROVISIONAL FILING

PROVISIONAL APPLICATION FOR LETTERS PATENT

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Susan C. Bock, residing at 2027 East 900 South, Salt Lake City, Utah 84108, have invented new and useful improvements in

**METHODS OF USING HIGH AFFINITY ATIII VARIANTS UNDER HIGH WALL
SHEAR RATE CONDITIONS**

for which the following is a specification.

METHODS OF USING HIGH AFFINITY ATIII VARIANTS UNDER HIGH WALL SHEAR RATE CONDITIONS

I. BACKGROUND

1. Antithrombin III (ATIII) is an endogenous inhibitor of the blood coagulation enzymes. ATIII inhibits activated coagulation factors such as thrombin and factor Xa, which participate in fibrin generation. Furthermore, rates of ATIII inhibition of activated coagulation factors are accelerated by binding to heparin and/or heparin sulfate proteoglycans (HSPGs). Disclosed herein variants of ATIII that preferentially bind heparin and HSPG under high shear rate conditions, such as when present in vasculature and mechanical vascular pieces, such as stents or tubing. Also disclosed are methods of using these variants in situations where high shear rates are present, as well as methods for identifying improved variants for heparin binding under high shear rate conditions.

II. SUMMARY

2. Disclosed are methods and compositions related to using variants of ATIII that have high affinity for heparin and heparin sulfate proteoglycans under high wall shear rate conditions.

III. BRIEF DESCRIPTION OF THE DRAWINGS

3. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments and together with the description illustrate the disclosed compositions and methods.

4. Figure 1 shows a diagram of an in vitro system for mimicking the conditions in a subjects circulatory system.

5. Figure 2 shows the amount and isoform of ATIII eluted from various sections of the tubing in the system shown in Figure 1 after fours incubation.

6. Figure 3 shows the amount of the alpha and beta isoforms of ATIII present on the tube of Figure 1 under different shear rates and over time.

7. Figure 4 shows the amount of alpha and beta ATIII present on the tube of Figure 1 along with the amount of binding of DES.N135A a mutant ATIII with higher binding affinity for heparin.

IV. DETAILED DESCRIPTION

8. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be

understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

9. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

10. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10" as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15.

11. References in the specification and concluding claims to parts by weight, of a particular element or component in a composition or article, denotes the weight relationship between the element or component and any other elements or components in the composition or article for which a part by weight is expressed. Thus, in a compound containing 2 parts by weight of component X and 5 parts by weight component Y, X and Y are present at a weight ratio of 2:5, and are present in such ratio regardless of whether additional components are contained in the compound.

12. A weight percent of a component, unless specifically stated to the contrary, is based on the total weight of the formulation or composition in which the component is included.

13. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

14. "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

15. "Primers" are a subset of probes which are capable of supporting some type of enzymatic manipulation and which can hybridize with a target nucleic acid such that the enzymatic manipulation can occur. A primer can be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art which do not interfere with the enzymatic manipulation.

16. "Probes" are molecules capable of interacting with a target nucleic acid, typically in a sequence specific manner, for example through hybridization. The hybridization of nucleic acids is well understood in the art and discussed herein. Typically a probe can be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art.

17. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

B. Compositions and methods

18. ATIII interacts with vasculature bound heparin and heparin sulfate proteoglycans (HSPGs). Disclosed herein ATIII binding to vascular wall heparin and heparan sulfate proteoglycans is important for maintaining patency in high wall-shear-rate regions of the circulatory system.

19.

20. The disclosed data indicate that ATIII – HSPG interactions are critically important under low and high shear rate conditions. High wall-shear-rate regions will become prone to thrombosis and inflammation when circulating β -ATIII concentrations drop to levels that do not support sufficient loading of vascular wall HSPG receptors, and ATIII-dependent surface anticoagulant, NF-Kb blocking and prostacyclin releasing activities are reduced. The disclosed high affinity heparin binding ATIII and low-dose super β -ATIIIs with enhanced-affinity for

heparin/HSPG will be useful for maximizing surface-bound ATIII in various high shear procedures such as angioplasty (with and without stent implantation), and during CPB and LVAD support.

21. Thrombotic and inflammatory reactions are significant clinical problems following balloon angioplasty with and without stent placement. Even using CBAS heparin-coated stents ~12% of patients in the Benestent II, Pani/Stent and Tosca trials still failed to achieve sustained patency (Kocsis, J., et al., (2000), *J. of Long-Term Effects of Medical Implants* 10, 19-45). Outcomes for these patients can be improved by a period of adjunctive treatment with super β -ATIII.

22. Another category of patients that can benefit from super β -ATIII treatment are those who have been implanted with non-pulsatile continuous flow centrifugal or impeller driven left ventricular assist devices that have heparin-coated interior surfaces. Approximately one third (8/22) of patients with Micromed DeBakey LVAD implants developed low blood flow rates and increased power consumption indicative of intrapump thrombosis, and required emergency thrombolytic treatment to restore flow (Rothenburger, M., et al., (2002), *Circulation* 106 [suppl II], I-189-192). Adjunctive treatment with low dose, enhanced-heparin-affinity super β -ATIIIs, especially during the period immediately following implantation, can be useful for boosting antithrombotic and anti-inflammatory activities on the patients own vascular surfaces and on surfaces of the device.

23. Heparin-coated circuits are widely employed in cardiovascular surgeries where on-pump CPB is utilized. In this context, adjunctive treatment with super β -ATIII could reduce thrombosis and associated neurocognitive function and stroke problems, and can also decrease intraoperative heparin requirements and hemorrhagic risk. On-pump bypass patients frequently develop ATIII deficiencies in conjunction with systemic inflammation and elastase activity increases (Cohen, J., et al., (1992), *J. Invest. Surg.* 5(45-9)). Disclosed are recombinant antithrombins that are highly resistant to inactivation by neutrophil elastase and cathepsin G, and these variants can also be made on a super β -ATIIIs backbone, which are being considered for use in settings that are inflammatory, as well as thrombotic.

24. Adjunctive super β -ATIII may be useful for improving the performance of CBAS-coated ePTFE grafts in low, as well as high, wall-shear-rate contexts. Although CBAS coating of ePTFE vascular grafts improved their performance in a canine carotid artery model, there is still room for improvement above the 50% patency rate observed at 180 days post implantation (Begovac, P., et al., (2003), *Eur. J. Vasc. and Endovasc. Surg.* 25, 432-437). The Fig. 4

experiment illustrates dramatic targeting of DES.N135A, a prototypically super beta ATIII, to the high wall-shear-rate section of the circuit. However, this enhanced-affinity ATIII also preferentially bound low wall shear- rate segments as well. This observation suggests additional potential benefits of super β -ATIII in applications targeting low wall-shear-rate targets.

25. Disclosed herein antithrombin III isoforms flowing through Carmeda CBAS tubing indicate that evolutionary conservation of the production of two glycoforms facilitates partitioning of ATIII antithrombotic and ant-inflammatory activities between the circulating blood and vascular wall surfaces. The higher heparin affinity of the β -ATIII isoform allows it to effectively bind to and protect low and high shear rate sections of the circulatory system, despite its relatively low concentration in blood. These findings provide paradigms for investigating and understanding ATIII – vascular surface interactions, as wells as strategies for the development of low dose super β ATIIIs to increase the antithrombotic and anti-inflammatory properties of vascular surfaces and heparin-coated medical devices.

26. The production of antithrombin III isoforms with different heparin/HSPG affinities is evolutionarily conserved. The β -ATIII isoform preferentially associates with HSPG receptors on vascular surfaces and may primarily mediate surface anticoagulant, antithrombotic and antiinflammatory reactions, whereas the principal function of the α -ATIII isoform may be to prevent stasis-associated (venous) thrombosis in the blood. These considerations suggest that β -ATIII interactions with vascular surfaces warrant further investigation, and in particular, that studies under physiologically realistic flowing conditions should be performed. Greater understanding of β -ATIII – vascular surface interactions may lead to the development of β -ATIII and β -ATIII derivative –based strategies to more effectively block pathologic thrombotic and inflammatory reactions on the vessel wall and in heparin-coated medical devices.

1. ATIII binds vasculature surfaces

27. It is generally believed that activated ATIII molecules bound on vascular surface HSPG receptors contribute substantively to the anticoagulant and antithrombotic properties of the endothelium (deAgostini, A., et al., (1990), *J. Cell Biol.* 111, 1293-1304). This view is supported by a recent report of lethal thrombosis in mice homozygous for an ATIII mutation that blocks binding to heparin/HSPG (Dewerchin, M., et al., (2003), *Circ. Res.* 93). Similarly, the high-affinity fraction of heparin that binds ATIII is required to prevent thrombosis in stents implanted in baboon extracorporeal arterio-venous shunts (Kocsis, J., et al., (2000), *J. of Long-Term Effects of Medical Implants* 10, 19-45). Therefore, antithrombin – vascular surface interactions play a critical role in maintaining circulatory system patency, and a previous contrary

report based on the non-thrombotic phenotype of mice deficient for 3-OST-1 (HajMohammadi, S., et al., (2003), *J. Clin. Invest.* 111, 989–999) may reflect redundancy of enzymes that mediate 3-O sulfation of the pentasaccharide sequence of HSPG (Weitz, J. (2003), *J. Clin. Invest.* 111, 952–954).

28. Although ATIII binding to vascular surfaces has been primarily investigated in the context of coagulation inhibition and thrombosis, recent studies indicate that this interaction also modulates anti-inflammatory properties of the endothelium. ATIII binding to HSPGs on endothelial cells or neutrophils promotes release of anti-inflammatory prostacyclin and blocks activation of proinflammatory NF-KB, which in turn leads to decreased platelet and neutrophil activation, chemotaxis, and interaction with the endothelium. These effects disappeared when the experiments were conducted with antithrombin that had been blocked in the heparin binding domain (Dunzendorfer, S., et al., (2001), *Blood* 97, 1079–1085; Hoffmann, J., et al., (2002), *Crit. Care Med.* 30, 218–225; Oelschläger, C., et al., (2002), *Blood* 99, 4015–4020).

2. Glycosylation isoforms of ATIII

29. Under no-flow conditions, differential glycosylation of the isoforms is responsible for a 6-fold difference in their affinity for the ATIII cofactor/receptor, heparin/HSPG (Turk, B., et al., (1997), *Biochemistry* 36, 6682–6691).

30. The origin of glycosylation differences between the α and β ATIII isoforms is synthetic rather than degradative. Partial modification of asparagine 135 occurs due to the presence of a serine, rather than a threonine, in the third position of its N-glycosylation consensus signal, leading to 50:50 production of α and β molecules (Picard, V., et al., (1995), *Biochemistry* 34, 8433–8440). The N-X-S tripeptide consensus sequence encoding the production of α and β glycoforms is conserved evolutionarily in vertebrates having 3 and 4 chambered hearts (mammals, birds, reptiles and amphibians) (Backovic, M., and Gettins, P. (2002), *J. Proteome Res.* 1, 367–373). This evolutionary conservation suggests that it is advantageous to carry two different antithrombin isoforms, and that each one has a distinct and critical function. In contrast, fish make only the β -ATIII isoform, which may be related to fundamental differences in fish circulatory systems, which have 2-chambered hearts.

31. Although α and β antithrombin are synthesized in a 50:50 ratio (Picard, V., et al., (1995), *Biochemistry* 34, 8433–8440, Bayston, T., et al., (1999), *Blood* 93, 4242–4247), they circulate in mammalian blood in a ratio of approximately 90 α :10 β . The β isoform clears more rapidly from the blood (Carlson, T., et al., (1985), *Biochem. J.* 225, 557–564) and occurs at

higher concentrations relative to α -ATIII in antithrombin eluted from rabbit aorta intima/media (Witmer, M., and Hatton, M. (1991), *Arteriosclerosis and Thrombosis* 11, 530-539). Moreover, β -ATIII, but not α -ATIII, reduced surface thrombin activity following balloon injury of rabbit aorta (Frebelius, S., et al., (1996), *Thromb. Vasc. Biol.* 16, 1292-1297).

32. Disclosed herein, experiments proving that the β -isoform preferentially associates with HSPG receptors of the vascular endothelium under high wall shear rates is the desired activity of binding the heparin and HSPG attached to walls. This data indicate that high affinity heparin binding, such as the β -isoform possesses, play an important role in anticoagulant, antithrombotic and anti-inflammatory reactions on these surfaces.

3. ATIII binds heparin and HSPG

33. The inhibitory activity of ATIII towards its target enzymes is enhanced by heparin (Rosenberg and Damus, (1973) *J. Biol. Chem.*, 248, 6490-6505) and vascular surface heparan sulfate proteoglycans (HSPGs) (Marcum *et al.*, (1983) *Am. J. Physiol.*, 245: H725-733). The heparin binding property of antithrombin directs ATIII to sites where its target enzymes are generated, and potentiates its activity on these surfaces. Thus heparin upregulates the inhibitory activity of ATIII, and also spatially regulates it so that highest rates of thrombin factor Xa inhibition are achieved on heparan sulfate proteoglycan (HSPG) – containing vascular surfaces.

4. ATIII is negatively regulated by proteases and elastases

34. ATIII is negatively regulated in part by elastases and proteases that cleave ATIII, preventing ATIII from inhibiting thrombin and factorXa. Human neutrophil elastase cleaves and inactivates ATIII (Jochum *et al.*, (1981). *Hoppe-Seyler's Z. Physiol. Chem.*, 362, 103-112). The reported neutrophil elastase cleavage sites were after the P5-Val and P4-Ile (Carrell and Owen, (1985) *Nature*, 317, 730-732). Furthermore, Jordan and colleagues showed that elastase inactivation of ATIII was heparin dependent (Jordan *et al.*, (1987) *Science*, 237, 777-779). It has been hypothesized that elevated elastase (Nuijens *et al.*, (1992) *J. Lab. Clin. Med.*, 119, 159-168) is responsible for the inactivation of ATIII in sepsis (Seitz *et al.*, (1987) *Eur. J. Haematol.*, 38, 231-240) and reduced antithrombin levels in septic disseminated intravascular coagulation (DIC) (Bick *et al.*, (1980) *Am. J. Clin. Path.*, 73, 577-583); (Buller and ten Cate, (1989) *Am. J. Med.*, 87, 44S-48S); (Damus and Wallace, (1989) *Thromb. Res.*, 6, 27); (Hellgren *et al.*, (1984a) *Intensive Care Med.*, 10, 23-28); (Lammle *et al.*, (1984) *Am J Clin Pathol*, 82, 396-404); (Mammen *et al.*, (1985) *Semin. Thromb. Hemost.*, 11, 373-383). Also included is the condition where cardiopulmonary bypass has increased elastase levels. Add cardiopulmonary bypass reference to end of this paragraph. (Cohen, J., Tenenbaum, N., Sarfati, I., Tyras, D., Graver, L.,

Weinstein, G., and Wise, L. (1992) In vivo inactivation of antithrombin III is promoted by heparin during cardiopulmonary bypass J. Invest. Surg. 5(45-9), which is herein incorporated by reference at least for material related to heparin and bypass surgery.

5. ATIII cleavage

35. Originally, the term protease referred to enzymes that cleaved the peptide bonds of low molecular weight polypeptides, and the term proteinase referred to enzymes that cleaved the peptide bonds of higher molecular weight proteins. More recently, the distinction between these two terms has become blurred in practical usage. In accordance with modern usage, this application also uses the term protease to refer to an enzyme that cleaves peptide bonds of proteins.

36. There are a variety of proteases that cleave the reactive loop of ATIII without the production of stable inhibitory complexes. These proteases can potentiate the expression of thrombin and fXa enzymatic activity by cleaving and inactivating the primary inhibitor of these coagulation factors, antithrombin III. Human neutrophil elastase (HNE) can cleave and inactivate ATIII. The primary cleavage sites for HNE are in the ATIII reactive loop, and their location can be described using the standard nomenclature of Schechter and Berger (Schechter, I, and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27:157-162, which is herein incorporated by reference at least for material related to ATIII cleavage and amino acid designations), wherein the amino acids of the reactive loop are referred to based on their location relative to the P1-P1' peptide bond that is cleaved by the thrombin or factor Xa during inhibitory complex formation. Residues amino terminal to this bond are designated P2, P3, etc, and those on C terminal to it are designated P2', P3', etc. HNE inactivates ATIII by cleavage after its P5-Val and P4-Ile residues (Carrell and Owen, (1985) *Nature*, 317, 730-732 which is herein incorporated by reference at least for material related to ATIII cleavage and amino acid designations).

37. Those of skill in the art understand that different allelic variants of ATIII and different species variants of ATIII for example, have an analogous site that is cleaved during inhibitory complex formation, and that this can readily be determined. Because the absolute position of this site in the numbered sequences of different ATIIIs may change, a standard nomenclature is employed to designate the relationship of reactive loop amino acids to the point of cleavage during inhibitory complex formation. (Schechter, I, and Berger, A. 1967. *Biochem. Biophys. Res. Commun.* 27:157-162).

6. Heparin/HSPG activation of ATIII

38. In the absence of activating cofactors, ATIIIs are less efficient inhibitors of the target enzymes. The basal rate of inhibition in the absence of cofactors is referred to as "progressive" activity. Second order rate constants for progressive ATIII inhibition of thrombin and factor Xa are typically in the 10^3 - 10^4 $M^{-1}sec^{-1}$ range. These rates, however, typically are accelerated by a factor of more than a thousand (i.e. into the 10^6 - 10^7 $M^{-1}sec^{-1}$ range) when certain kinds of sulfated glycosaminoglycan cofactors (heparin or heparan sulfate proteoglycans (HSPG)) bind to ATIII. Heparin is a widely used pharmaceutical that has been administered as an anticoagulant since the 1940s, while heparan sulfate proteoglycans (HSPGs) serve as the physiological cofactor for ATIII. HSPGs anchored to the vessel wall and matrix present heparin-like molecules to circulating blood and serve to localize and activate ATIII on surfaces where coagulation enzymes are generated.

7. Types of ATIII

a) ATIIIs with high affinity for bound heparin or HSPGs

39. It is desirable to have ATIIIs which bind heparin or HSPGs with high affinity when the HSPG or heparin are bound to a surface, such as a vasculature surface or a mechanical surface, such as a stent or tubing.

40. For example, the measurements of α , β and recombinant antithrombin K_{AS} for surface bound heparin at different shear rates, disclosed herein, are necessary for developing an improved and more physiologically accurate understanding of ATIII-HSPG regulatory interactions. In addition, knowledge of how ATIII binding affinities vary over the range of physiologically and pathologically relevant flow conditions will expedite the design and development of strategies utilizing low-dose, high-affinity recombinant antithrombins to efficiently block thrombin generation and inflammatory reactions on vascular and biomedical device surfaces. The calculations in Table 3 show that by using 0.8 mm id CBAS heparin-coated PVC tubing and reasonable amounts of antithrombins, K_{AS} can be measured by equilibrium binding and dissociation and association rate methods for wall-shear-rates in the 50 – 3500 sec^{-1} range.

41. Table 3 Calculation of tubing id and length for K_d studies

wall-shear-rate = $\gamma_w = 4Q / \pi R^3$

Q = volumetric flow rate, R = radius at wall of tube

pump tubing id Q range, ml/min	1.6 mm 05 – 2	3.2 mm 0.5 - 15		
CBAS id mm	radius mm	Q ml/min	Q mm^3/sec	γ_w sec^{-1}
0.8	0.4	0.2	3	66
0.8	0.4	2	33	663

0.8	0.4	5	83	1659
0.8	0.4	12	200	3981

shear rates in Ruggieri platelet studies: 50, 630, 1500 sec⁻¹

highest shear rates in normal circulation = arterioles: 500 - 5000 sec⁻¹

shear rates at top of plaques in 50% occluded artery: 3000-10,000 sec⁻¹

5

42. The K_{AS} for any ATIII molecule and the surface bound heparin or HSPG can be achieved by complementary equilibrium binding and association/dissociation rate strategies. The K_{AS} can be measured at wall-shear-rates of, for example, 50, 630 and 1500 sec⁻¹, which are the standard values used by Ruggieri's group for investigations of platelet adhesion under flow. Measurements can also be conducted at, for example, 3500 sec⁻¹ since wall-shear-rates of this magnitude have been measured in 50% stenosed arteries, which are also of interest as potential super β -ATIII targets. The disclosed calculations indicate that rheologically relevant K_{AS} measurements can be obtained using 0.8 mm id CBAS tubing and quantities of plasma derived ATIII isoforms and recombinant ATIIIs that we can realistically produce (see Table 3).

43. Certain variant ATIIIs with increased affinity for heparin are disclosed in United States Patent No. 5,700,663, which is herein incorporated by reference at least for ATIII variants. Variants disclosed are those that contain amino acid substitutions at position 49, 96, 135, 155, 192, 393, or 394 of SEQ ID NO:1.

44. Certain other variant ATIIIs with increased affinity for heparin are disclosed in United States Patent No. 5,420,252 which is herein incorporated by reference at least for ATIII variants. Variants disclosed are those that contain amino acid substitutions in ATIII at positions 11 to 14, 41 to 47, 125 to 133, and 384 to 398 are substituted by another amino acid(s) such as Ala, Gly, Trp, Pro, Leu, Val, Phe, Tyr, Ile, Glu, Ser, Gln, Asn and Arg.

45. Other variants of ATIII disclosed herein are variants that have improved elastase and/or protease resistance while still retaining thrombin and/or fXa inhibition. These variants can also have improved heparin binding. These mutants can be found in United States Patent Application nos 60/085,197, 60/384599, 09/305588, 10/014,658, and PCT applications PCT/US99/10549 and PCT/US03/17506, which are herein incorporated by reference at least for material related to variants of ATIII. The disclosed variants include Disclosed are substitutions, wherein the substitution made at position P2, alone or collective with substitutions at either P3, P4, P5, P6, P7, and/or P8 or any other variants disclosed herein, is P.

46. Disclosed are substitutions, wherein the substitution made at position P3, alone or collective with substitutions at either P2, P4, P5, P6, P7, and/or P8 or any other variants disclosed herein, is D, E, G, H, I, K, L, N, P, Q, R, S, W, or Y.

47. Disclosed are substitutions, wherein the substitution made at position P3, alone or collective with substitutions at either P2, P4, P5, P6, P7, and/or P8 or any other variants disclosed herein, is D, E, H, K, L, P, Q, R, W, or Y.

48. Disclosed are substitutions, wherein the substitution made at position P4, alone or collective with substitutions at either P2, P3, P5, P6, P7, and/or P8 or any other variants disclosed herein, is A, F, G, L, N, P, Q, V, or W..

49. Disclosed are substitutions, wherein the substitution made at position P4, alone or collective with substitutions at either P2, P3, P5, P6, P7, and/or P8 or any other variants disclosed herein, is L, N, Q, V, or W.

50. Disclosed are substitutions, wherein the substitution made at position P5, alone or collective with substitutions at either P2, P3, P4, P6, P7, and/or P8 or any other variants disclosed herein, is E, F, G, P, D, S, T, N, Q, H, R, K, or V.

51. Disclosed are substitutions, wherein the substitution made at position P6, alone or collective with substitutions at either P2, P3, P4, P5, P7, and/or P8 or any other variants disclosed herein, is E, G, L, or T.

52. Disclosed are substitutions, wherein the substitution made at position P7, alone or collective with substitutions at either P2, P3, P4, P5, P6, and/or P8 or any other variants disclosed herein, is E, N, Q, V, L, F, S, T, or H.

53. Disclosed are substitutions, wherein the substitution made at position P8, alone or collective with substitutions at either P2, P3, P4, P5, P6, P7, and/or P8, or any other variants disclosed herein, is E.

54. Disclosed are variants, having at least one substitution at position P2, P3, P4, P5, P6, or P7, wherein the substitution at P2 can be P, wherein the substitution at P3 can be D, E, G, H, I, K, L, N, P, Q, R, S, W, or Y, wherein the substitution at P4 can be A, F, G, L, N, P, Q, V, or W, wherein the substitution at P5 can be E, F, G, or P, wherein the substitution at P6 can be E, G, L, or T, wherein the substitution at P7 can be E or Q.

55. Disclosed are variants, having at least one substitution at position P2, P3, P4, P5, P6, or P7, wherein the substitution at P2 can be P, wherein the substitution at P3 can be D, E, H, K, L, P, Q, R, W, or Y, wherein the substitution at P4 can be L, N, Q, V, or W, wherein the

substitution at P5 can be E or F wherein the substitution at P6 can be G or L, wherein the substitution at P7 can be E.

56. Disclosed are variants, having at least one substitution at position P2, P3, P4, P5, P6, or P7, wherein the substitution at P2 can be P, wherein the substitution at P3 can be D, E, G, H, I, K, L, N, P, Q, R, S, W, or Y, wherein the substitution at P4 can be A, F, G, L, N, P, Q, V, or W, wherein the substitution at P5 can be D, E, F, G, H, K, N, P, Q, R, S, T, or V wherein the substitution at P6 can be E, G, L, or T, wherein the substitution at P7 can be E, F, H, I, L, N, Q, S, T, or V, or wherein the substitution at P8 can be E.

57. Disclosed are variants, having at least one substitution at position P2, P3, P4, P5, P6, or P7, wherein the substitution at P2 can be P, wherein the substitution at P3 can be D, E, H, K, L, P, Q, R, W, or Y, wherein the substitution at P4 can be L, N, Q, V, or W, wherein the substitution at P5 can be D, H, K, N, Q, R, S, T, or V wherein the substitution at P6 can be G or L, wherein the substitution at P7 can be F, H, L, S, T, V.

58. Disclosed are variants, having at least one substitution at position P2, P3, P4, P5, P6, or P7, wherein substitution at P2 can be P, wherein the substitution at P3 can be D, E, H, K, L, P, Q, R, W, or Y, wherein the substitution at P4 can be L, N, Q, V, or W.

59. Disclosed are variants, having at least one substitution at position P7 or P5, wherein the substitution at P7 can be G, V, L, F, S, T, N, Q, H, R, or, K, and wherein the substitution at P5 can be D, S, T, N, Q, H, R, K, V, or G.

60. Disclosed are variants, having at least one substitution at position P7 or P5, wherein the substitution at P7 can be E, Q, V, L, F, S, T, H, or E, and wherein the substitution at P5 can be E, F, G, P, D, S, T, N, Q, H, R, K, or V.

61. Disclosed are variants of antithrombin III, comprising a substitution at position P2, wherein the substitution at P2 is a P, along with at least one other substitution disclosed herein.

62. Disclosed are variants of antithrombin III, comprising a substitution at position P3, wherein the substitution at P3 is a D, E, H, K, L, P, Q, R, W, or Y.

63. Disclosed are variants of antithrombin III, comprising a substitution at position P4, wherein the substitution at P4 is a L, N, Q, V, or W, and when the substitution of W occurs with at least one other substitution disclosed herein.

64. Disclosed are variants of antithrombin III, comprising at least one substitution at either position P3 and P4, wherein the substitution at P3 is D, E, H, K, L, P, Q, R, W, or Y, and wherein the substitution at P4 is L, N, Q, V, or W, and at least one substitution at P2, P5, P6, P7,

and P8, wherein the substitution at P2 is P, P5 is E, F, G, or P, wherein the substitution at P6 is E, G, L, or T, wherein the substitution at P7 is E or Q, and wherein the substitution at P8 is E.

65. Disclosed are variants of antithrombin III, comprising at least two substitutions at P3 and P4, wherein the substitution at P3 is D, E, G, H, I, K, L, N, P, Q, R, S, W, or Y, and wherein
5 the substitution at P4 is L, N, Q, V, or W.

66. Disclosed are variants of antithrombin III, comprising at least two substitutions at either position P3 and P4, wherein the substitution at P3 is D, E, H, K, L, P, Q, R, W, or Y, and wherein the substitution at P4 is A, F, G, L, N, P, Q, V, or W.

67. Disclosed are variants of antithrombin III, comprising a substitution at least two
10 substitutions at P2, P3 and P4, wherein the substitution at P2 is P, wherein the substitution at P3 is D, E, G, H, I, K, L, N, P, Q, R, S, W, or Y, and wherein the substitution at P4 is A, F, G, L, N, P, Q, V, or W.

68. Disclosed are variants of antithrombin III, comprising a substitution at least one substitution at P2, P3 and P4, wherein the substitution at P2 is P, wherein the substitution at P3
15 is D, E, H, K, L, P, Q, R, S, W, or Y, and wherein the substitution at P4 is L, N, Q, V, or W.

69. Disclosed are variants of antithrombin III, comprising a substitution at least one substitution at P3 and P4, wherein the substitution at P3 is D, E, H, K, L, P, Q, R, S, W, or Y, and wherein the substitution at P4 is L, N, Q, V, or W.

70. Disclosed are variants of antithrombin III, wherein the variant antithrombin III has a
20 combined activity greater than or equal to plasma ATIII in a coupled assay.

71. Disclosed are variants of antithrombin III, wherein the variant antithrombin III has a combined activity greater than or equal to 2, 5, or 10, times the activity of plasma ATIII in a coupled assay.

72. Disclosed are variants of antithrombin III, wherein the variant antithrombin III has an
25 increased protease resistance.

73. Also disclosed are variants in Olson ST, Frances-Chmura AM, Swanson R, Bjork I, Zettlmeissl G. "Effect of individual carbohydrate chains of recombinant antithrombin on heparin affinity and on the generation of glycoforms differing in heparin affinity," Arch Biochem Biophys. 1997 May 15;341(2):212-21; Bjork I, Ylinenjarvi K, Olson ST, Hermentin P, Conradt
30 HS, Zettlmeissl G., "Decreased affinity of recombinant antithrombin for heparin due to increased glycosylation," Biochem J. 1992 Sep 15;286 (Pt 3):793-800; and Garone L, Edmunds T, Hanson E, Bernasconi R, Huntington JA, Meagher JL, Fan B, Gettins PG., "Antithrombin-heparin affinity reduced by fucosylation of carbohydrate at asparagine 155," Biochemistry. 1996 Jul

9;35(27):8881-9, all of which are herein incorporated by reference at least for material related to variant ATIII and their sequence and structure.

74. Also disclosed are variants of ATIII and the use of these variants where the Kd for heparin is less than or equal to 0.01nM, 0.05nM, 0.1nM, 0.5nM, 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 7 nM, 8 nM, 9 nM, 10 nM, 11 nM, 12 nM, 13 nM, 14 nM, 15 nM, 16 nM, 17 nM, 18 nM, 19 nM, 20 nM, 21 nM, 22 nM, 23 nM, 24 nM, 25 nM, 26 nM, 27 nM, 28 nM, 29 nM, 30 nM, 31 nM, 32 nM, 33 nM, 34 nM, 35 nM, 36 nM, 37 nM, 38 nM, 39 nM, 40 nM, 41 nM, 42 nM, 43 nM, 44 nM, 45 nM, 46 nM, 47 nM, 48 nM, 49 nM, 50 nM, 51 nM, 52 nM, 53 nM, 54 nM, 55 nM, 56 nM, 57 nM, 58 nM, 59 nM, 60 nM, 61 nM, 62 nM, 63 nM, 64 nM, 65 nM, 66 nM, 67 nM, 68 nM, 69 nM, 70 nM, 71 nM, 72 nM, 73 nM, 74 nM, 75 nM, 76 nM, 77 nM, 78 nM, 79 nM, 80 nM, 81 nM, 82 nM, 83 nM, 84 nM, 85 nM, 86 nM, 87 nM, 88 nM, 89 nM, 90 nM, 91 nM, 92 nM, 93 nM, 94 nM, 95 nM, 96 nM, 97 nM, 98 nM, 99 nM, 100 nM, 101 nM, 102 nM, 103 nM, 104 nM, 105 nM, 106 nM, 107 nM, 108 nM, 109 nM, 110 nM, 111 nM, 112 nM, 113 nM, 114 nM, 115 nM, 116 nM, 117 nM, 118 nM, 119 nM, 120 nM, 121 nM, 122 nM, 123 nM, 124 nM, 125 nM, 126 nM, 127 nM, 128 nM, 129 nM, 130 nM, 131 nM, 132 nM, 133 nM, 134 nM, 135 nM, 136 nM, 137 nM, 138 nM, 139 nM, 140 nM, 141 nM, 142 nM, 143 nM, 144 nM, 145 nM, 146 nM, 147 nM, 148 nM, 149 nM, 150 nM, 151 nM, 152 nM, 153 nM, 154 nM, 155 nM, 156 nM, 157 nM, 158 nM, 159 nM, 160 nM, 161 nM, 162 nM, 163 nM, 164 nM, 165 nM, 166 nM, 167 nM, 168 nM, 169 nM, 170 nM, 171 nM, 172 nM, 173 nM, 174 nM, 175 nM, 176 nM, 177 nM, 178 nM, 179 nM, 180 nM, 181 nM, 182 nM, 183 nM, 184 nM, 185 nM, 186 nM, 187 nM, 188 nM, 189 nM, 190 nM, 191 nM, 192 nM, 193 nM, 194 nM, 195 nM, 196 nM, 197 nM, 198 nM, 199 nM, 200 nM, 201 nM, 202 nM, 203 nM, 204 nM, 205 nM, 206 nM, 207 nM, 208 nM, 209 nM, 210 nM, 211 nM, 212 nM, 213 nM, 214 nM, 215 nM, 216 nM, 217 nM, 218 nM, 219 nM, 220 nM, 221 nM, 222 nM, 223 nM, 224 nM, 225 nM, 226 nM, 227 nM, 228 nM, 229 nM, 230 nM, 231 nM, 232 nM, 233 nM, 234 nM, 235 nM, 236 nM, 237 nM, 238 nM, 239 nM, 240 nM, 241 nM, 242 nM, 243 nM, 244 nM, 245 nM, 246 nM, 247 nM, 248 nM, 249 nM, 250 nM, 251 nM, 252 nM, 253 nM, 254 nM, 255 nM, 256 nM, 257 nM, 258 nM, 259 nM, 260 nM, 261 nM, 262 nM, 263 nM, 264 nM, 265 nM, 266 nM, 267 nM, 268 nM, 269 nM, 270 nM, 271 nM, 272 nM, 273 nM, 274 nM, 275 nM, 276 nM, 277 nM, 278 nM, 279 nM, 280 nM, 281 nM, 282 nM, 283 nM, 284 nM, 285 nM, 286 nM, 287 nM, 288 nM, 289 nM, 290 nM, 291 nM, 292 nM, 293 nM, 294 nM, 295 nM, 296 nM, 297 nM, 298 nM, 299 nM, 300 nM, 301 nM, 302 nM, 303 nM, 304 nM, 305 nM, 306 nM, 307 nM, 308 nM, 309 nM, 310 nM, 311 nM, 312 nM, 313 nM, 314 nM, 315 nM, 316 nM, 317 nM, 318 nM, 319 nM, 320 nM, 321 nM, 322

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75. It is also understood that ATIIIs which are produced in insect expression systems produce ATIIIs having higher affinities for heparin than ATIIIs produced in other recombinant systems or from native plasma. For example, ATIIIalpha from Hamster Cho expression system has a Kd of 63 nM and a Kd of beta of 18 nM. The Kds of alpha and beta from an insect system are 8 and 1 nM respectively. This is because the insect expressed ATIIIs have smaller glycosylation chains than the other expression systems and other than plasma ATIII. These smaller chains aid in binding and high affinity binding structure formation.

76. Also as the shear rate increases the effective Kd of binding wall bound heparin decreases more quickly for alpha ATIII than for beta ATIII.

8. Types conditions

a) Shear rate ranges

77. The disclosed compositions and methods are related to the shear rate that occurs at the surface of, for example, blood vessels, or tubing through which body fluids flow. The shear rate is related to the diameter and the flow rate of the liquid flowing through the vessel or tube. Shear rate is determined as disclosed herein. The disclosed methods in certain embodiments

include conditions where shear rates of at least 20 sec⁻¹, 40 sec⁻¹, 60 sec⁻¹, 80 sec⁻¹, 100 sec⁻¹, 200 sec⁻¹, 300 sec⁻¹, 400 sec⁻¹, 500 sec⁻¹, 600 sec⁻¹, 700 sec⁻¹, 800 sec⁻¹, 900 sec⁻¹, 1000 sec⁻¹, 1100 sec⁻¹, 1200 sec⁻¹, 1300 sec⁻¹, 1400 sec⁻¹, 1500 sec⁻¹, 1600 sec⁻¹, 1700 sec⁻¹, 1800 sec⁻¹, 1900 sec⁻¹, 2000 sec⁻¹, 2100 sec⁻¹, 2200 sec⁻¹, 2300 sec⁻¹, 2400 sec⁻¹, 2500 sec⁻¹, 2600 sec⁻¹, 2700 sec⁻¹, 2800 sec⁻¹, 2900 sec⁻¹, 3000 sec⁻¹, 3100 sec⁻¹, 3200 sec⁻¹, 3300 sec⁻¹, 3400 sec⁻¹, 3500 sec⁻¹, 3600 sec⁻¹, 3700 sec⁻¹, 3800 sec⁻¹, 3900 sec⁻¹, or 4000 sec⁻¹ are present.

b) Time of exposure

78. Also disclosed are embodiments of the methods and compositions where the conditions present include varying times of exposure. For example, disclosed are conditions where the compositions are added by bolus or are added by continuous infusion.

c) Infusion location

79. Disclosed are methods of administration of ATIII, specifically ATIII, with increased affinity for bound heparin and HSPG under low and high shear rate conditions as disclosed herein. In certain embodiments, the administration is administered immediately upstream of the area where ATIII loading is desired, such as a stent. It is shown herein that there is an improved effect of ATIII binding to a heparin or HSPG loaded surface when the ATIII is injected near, upstream of the desired ATIII binding location. For example, in certain embodiments, administration of the ATIII, such as a high affinity ATIII, such as an ATIII having the properties of a beta ATIII, as disclosed herein, through the catheter during or after an angioplasty procedure or through the catheter after the placing of the stent, just upstream of the stent or angioplasty site is a preferable mode of ATIII drug administration.

9. Types of systems

80. The disclosed compositions and methods can be used in many different types of systems. For example, the disclosed compositions and methods can be used in a subject in vivo, where the system is the vasculature of the subject, such as the arterial and venous vessels. For example, the compositions and methods can be used in this way after or during an angioplasty procedure. The methods and compositions can also be used in other systems, including heart pumps and stents.

81. Typically the systems are related in that they include heparin or HSPG attached to a solid surface, such as a vessel or a tube, or metal stent. Thus, systems that are coated with heparin are disclosed systems.

10. Materials coated with heparin

82. There are many different materials and devices that contain heparin and heparin sulfate proteoglycans which bear pentasaccharide structures, can be placed on. One type of technology for placing heparin on surfaces is CBASTM technology (Larm O. et al., Biomaterials Med. Devices Artif. Organs (1983) 11:161, which is herein incorporated by reference at least for material related to heparin loading of surfaces and methods for performing the same). CBASTM has been used to coat heparin on heart lung bypass machines (Cardiatech Inc.), stents, CBASTM tubing, CBASTM, instech labs, heparin-coated extracorporeal circuits, and cardiopulmonary bypass machines and procedures (CPB). Various devices such as blood oxygenator circuits for blood oxygenation have been coated with CBASTM technology produced by, for example, Medtronic Inc. Ventricular assist devices have been coated and produced by, for example, Berlin Heart Inc. Vascular grafts have been coated and produced by, for example, Gore Inc. Coronary stents have been coated and produced by, for example, Cordisc Inc. Central venous catheters have been coated and produced by, for example, CCL Inc. Intraarterial blood gas sensors have been coated and produced by, for example, Diametrics Inc. Continuous blood sampling catheters have been coated and produced by, for example, Carmeda Inc. Intraocular lens have been coated and produced by, for example, Pharmacia-Upjohn Inc.

83. CBASTM can be used to coat heparin on thermoplastics, such as Delrin (DuPont), nylon, polycarbonate, polyethylene, polysulfone, polyurethane, and PVC; Rubbers, such as silicone and latex; metals, such as titanium, stainless steel, nitinol; wovens; filter media, such as glass; and Pet.

84. There are other technologies capable of loading heparin onto a surface. These technologies are discussed in Andersson J. et al., J. Bio Med. Materials Research (2003) 67A(2):458 which is herein incorporated at least for material related to heparin coating and different means for performing the same. For example, there is a the hepamed technique developed by Medtronics and the Curline Heparine Surface technique (vanDerGeissen W.J. Curr. Interv. Cardiology Rep. (1999) 1:234. (which is herein incorporated by reference at least for material related to heparin loading of surfaces and methods for performing the same)

85. Thus, any device or material can have heparin coated on it, by any available means.

86. The disclosed relationship between binding vessel bound heparin and HSPG provides direction as to the concentration of how much heparin or HSPG to coat a device with. The relationship between the naturally occurring binding of the beta isoform of ATIII to a vessel

bound heparin or HSPG rather than the alpha isoform is rooted in the differential binding affinities that each have for bound heparin or HSPG, which change under different shear rates.

87. There are many devices and materials that can have heparin or HSPG coated on them, and these devices and materials are typically brought into contact with the blood of a subject.

5 Devices and materials that are brought into contact with the blood of a subject can be divided into two categories, 1) devices and materials which will be in continuous contact with the blood of the subject because they are being implanted in the subject and are intended for long term use, such as a coronary stent, and 2) devices and materials which are considered transient because the blood is simply circulating through the system for period of time, such as a heart lung machine.

10 The discoveries disclosed herein, that there is an evolutionarily conserved relationship between the alpha and beta forms of ATIII and that this relationship has a very distinct purpose in partitioning one isoform of ATIII to vessel walls and one isoform to circulate in the free flowing blood, provides direction as to the type of heparin placement needed for a) systems that are approximating a blood circulatory system, such as a heart lung machine, and b) systems that are
15 more long term, such as implants.

88. In other situations, when for example, the device is to be considered an implant, such as a coronary stent, a different goal can be set based on the disclosed evolutionarily conserved relationship. It is disclosed herein, that under low and high shear rate conditions, the alpha form is circulating in the blood, because it has an affinity for bound heparin or HSPG which is much
20 lower than the affinity of beta isoform. This means that the endogenous ATIII, that is circulating in the system, is of little use for binding the implanted device, such as the stent. This means that the desired effective concentration of heparin on these types of devices, is actually greater than the K_d of alpha ATIII for heparin, or greater than about 300nM. By making devices, such as stents with concentrations of heparin greater than about 300nM, the stent or device can take
25 advantage of the subjects endogenous floating alpha ATIII, and get a higher binding of the heparin or HSPG on the stent or device by the alpha ATIII.

89. Thus, disclosed are devices with the concentrations of heparin and HSPG tailored as discussed herein to take advantage of the evolutionarily conserved relationship between the alpha and beta ATIII heparin binding, as well as methods of making these devices, and methods of
30 using these devices using methods well known. For example, the devices can be made using CBASTM technology.

11. Methods of identifying molecules that bind heparin/HSPGs with high affinity in high wall shear rate conditions

90. Disclosed are methods for identifying super β -ATIII that have enhanced affinity for vascular wall HSPG receptors and heparin-coated biomaterials, and that are resistant to inflammatory inactivation (wild type ATIII is extremely sensitive to cleavage and inactivation by neutrophil elastase). The structural basis of ATIII heparin binding and activation is discussed in
 5 for example, Olson, S., et al., (2002), *Trends Cardiovasc. Med.* **12**, 198-205; Jairajpuri, M., et al., (2002), *J. Biol. Chem.* **277**, 24460-5; and Jairajpuri, M., et al., (2003), *J. Biol. Chem.* **278**, 15941-50. In certain embodiments, the super β -ATIII will have affinities greater than those published in Jairajpuri, M., et al., (2002), *J. Biol. Chem.* **277**, 24460-5; Ersdal-Badju, E., et al., (1995), *Biochem. J.* **310**, 323-330, US 5,420,252 (Kato); US 5,618,713 and 5,700,663
 10 (Zettlemessl) high-heparin-affinity antithrombins.

91. Also disclosed are high-heparin-affinity elastase- and cathepsin G - resistant antithrombins for use in acute inflammatory environments (United States Patent Applications 60/085,197, 60/384599, 09/305588, 10/014,658, and PCT applications PCT/US99/10549 and PCT/US03/17506, which are herein incorporated by reference at least for material related to
 15 variant ATIII, including specific sequences of variant ATIII).

92. The disclosed methods take advantage of the information that it is preferred that ATIII bind heparin when it is bound to a vessel wall, and/or under high shear conditions. The disclosed systems, such as the in vitro circulatory system disclosed in figure 1, can be used to screen various ATIII variants for activity. For example, a variant can be tested whether it binds
 20 the high shear rate section of the tube can be determined as disclosed herein. Variants of ATIII can be made using any standard means of introducing variation into a sequence, such as discussed herein, for example, using PCR mutagenesis. It is understood that these mutations can be made on top of the mutations specifically already disclosed herein which, for example, increase heparin binding affinity or elastase or protease resistance. It is also understood that traditional
 25 binding assays and screening methods can also be employed to isolate ATIII or other molecules such as functional nucleic acids or monoclonal or polyclonal antibodies, which bind heparin in the way that ATIII binds heparin under low and high shear rate conditions.

93. It is understood that also disclosed are methods of making molecules that can be identified as described herein, by, for example, synthesizing the identified molecules. Also
 30 disclosed are the molecules which are so identified as well as methods of using these molecules.

12. Method of coadministration of heparin and ATIII

94. Disclosed herein are methods of coadministration of ATIII and heparin in any form after, for example, a coronary angioplasty procedure or placement of a coronary stent. It is

preferred that the ATIII, be high affinity ATIII, such as beta ATIII or other ATIIIs as disclosed herein.

C. Compositions

95. Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular ATIII is disclosed and discussed and a number of modifications that can be made to a number of molecules including the ATIII are discussed, specifically contemplated is each and every combination and permutation of ATIII and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

1. Characteristics and techniques for biological macromolecules such as proteins and nucleic acids

96. There are a number of properties and characteristics of biological macromolecules, such as sequence similarities, hybridizations, sequence variation, and so forth that are applicable to the disclosed ATIII and other molecules.

a) Sequence similarities

97. It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining

homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

98. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

99. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

100. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

101. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is

calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

b) Hybridization/selective hybridization

102. The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

103. Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the T_m (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the T_m . The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on

filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 1987:154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

104. Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their K_d , or where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their K_d .

105. Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the

primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

106. Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

107. It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

c) Nucleic acids

108. There are a variety of molecules disclosed herein that are nucleic acid based, including for example the nucleic acids that encode, for example, ATIII as well as any other proteins disclosed herein, as well as various functional nucleic acids. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantageous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

(I) Nucleotides and related molecules

109. A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymine-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a nucleotide is pentavalent phosphate. A non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

110. A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to nucleotides are well known in the art and would include for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, and 2-aminoadenine as well as modifications at the sugar or phosphate
5 moieties.

111. Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate
10 moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

112. It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid
15 moieties such as a cholesterol moiety. (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989,86, 6553-6556),

113. A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a
20 purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

114. A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH2 or O) at the C6 position of
25 purine nucleotides.

(2) Sequences

115. There are a variety of sequences related to, for example, ATIII, as well as any other protein disclosed herein that are disclosed on Genbank, and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences
30 contained therein.

116. A variety of sequences are provided herein and these and others can be found in Genbank, at www.pubmed.gov. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular

sequence to other related sequences. Primers and/or probes can be designed for any sequence given the information disclosed herein and known in the art.

(3) Primers and probes

117. Disclosed are compositions including primers and probes, which are capable of interacting with the genes disclosed herein. In certain embodiments the primers are used to support DNA amplification reactions. Typically the primers will be capable of being extended in a sequence specific manner. Extension of a primer in a sequence specific manner includes any methods wherein the sequence and/or composition of the nucleic acid molecule to which the primer is hybridized or otherwise associated directs or influences the composition or sequence of the product produced by the extension of the primer. Extension of the primer in a sequence specific manner therefore includes, but is not limited to, PCR, DNA sequencing, DNA extension, DNA polymerization, RNA transcription, or reverse transcription. Techniques and conditions that amplify the primer in a sequence specific manner are preferred. In certain embodiments the primers are used for the DNA amplification reactions, such as PCR or direct sequencing. It is understood that in certain embodiments the primers can also be extended using non-enzymatic techniques, where for example, the nucleotides or oligonucleotides used to extend the primer are modified such that they will chemically react to extend the primer in a sequence specific manner. Typically the disclosed primers hybridize with the nucleic acid or region of the nucleic acid or they hybridize with the complement of the nucleic acid or complement of a region of the nucleic acid.

d) Nucleic Acid Delivery

118. In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the disclosed nucleic acids can be in the form of naked DNA or RNA, or the nucleic acids can be in a vector for delivering the nucleic acids to the cells, whereby the antibody-encoding DNA fragment is under the transcriptional regulation of a promoter, as would be well understood by one of ordinary skill in the art. The vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the disclosed

nucleic acid or vector can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

119. As one example, vector delivery can be via a viral system, such as a retroviral
 5 vector system which can package a recombinant retroviral genome (see e.g., Pastan et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:4486, 1988; Miller et al., *Mol. Cell. Biol.* 6:2895, 1986). The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells nucleic acid encoding a broadly neutralizing antibody (or active fragment thereof). The exact method of introducing the altered nucleic acid into mammalian cells is, of course, not limited to the use of
 10 retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors (Mitani et al., *Hum. Gene Ther.* 5:941-948, 1994), adeno-associated viral (AAV) vectors (Goodman et al., *Blood* 84:1492-1500, 1994), lentiviral vectors (Naidini et al., *Science* 272:263-267, 1996), pseudotyped retroviral vectors (Agrawal et al., *Exper. Hematol.* 24:738-747, 1996). Physical transduction techniques can also be used, such as liposome delivery
 15 and receptor-mediated and other endocytosis mechanisms (see, for example, Schwartzberger et al., *Blood* 87:472-478, 1996). This disclosed compositions and methods can be used in conjunction with any of these or other commonly used gene transfer methods.

120. As one example, if the antibody-encoding nucleic acid is delivered to the cells of a subject in an adenovirus vector, the dosage for administration of adenovirus to humans can
 20 range from about 10^7 to 10^9 plaque forming units (pfu) per injection but can be as high as 10^{12} pfu per injection (Crystal, *Hum. Gene Ther.* 8:985-1001, 1997; Alvarez and Curiel, *Hum. Gene Ther.* 8:597-613, 1997). A subject can receive a single injection, or, if additional injections are necessary, they can be repeated at six month intervals (or other appropriate time intervals, as determined by the skilled practitioner) for an indefinite period and/or until the efficacy of the
 25 treatment has been established.

121. Parenteral administration of the nucleic acid or vector, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid
 solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration
 30 involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein. For additional discussion of suitable formulations and various routes of administration of therapeutic

compounds, see, e.g., *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995.

e) Expression systems

122. The nucleic acids that are delivered to cells typically contain expression
controlling systems. For example, the inserted genes in viral and retroviral systems usually
contain promoters, and/or enhancers to help control the expression of the desired gene product.
A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed
location in regard to the transcription start site. A promoter contains core elements required for
basic interaction of RNA polymerase and transcription factors, and may contain upstream
elements and response elements.

(1) Viral Promoters and Enhancers

123. Preferred promoters controlling transcription from vectors in mammalian host
cells may be obtained from various sources, for example, the genomes of viruses such as:
polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most
preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin :
promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40
restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., Nature,
273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently
obtained as a HindIII E restriction fragment (Greenway, P.J. et al., Gene 18: 355-360 (1982)).
Of course, promoters from the host cell or related species also are useful herein.

124. Enhancer generally refers to a sequence of DNA that functions at no fixed
distance from the transcription start site and can be either 5' (Laimins, L. et al., Proc. Natl. Acad. Sci. 78: 993 (1981)) or 3' (Lusky, M.L., et al., Mol. Cell Bio. 3: 1108 (1983)) to the
transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., Cell 33:
729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., Mol. Cell Bio. 4:
1293 (1984)). They are usually between 10 and 300 bp in length, and they function in cis.
Enhancers function to increase transcription from nearby promoters. Enhancers also often
contain response elements that mediate the regulation of transcription. Promoters can also
contain response elements that mediate the regulation of transcription. Enhancers often
determine the regulation of expression of a gene. While many enhancer sequences are now
known from mammalian genes (globin, elastase, albumin, -fetoprotein and insulin), typically
one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples
are the SV40 enhancer on the late side of the replication origin (bp 100-270), the

cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

125. The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

126. In certain embodiments the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In certain constructs the promoter and/or enhancer region be active in all eukaryotic cell types, even if it is only expressed in a particular type of cell at a particular time. A preferred promoter of this type is the CMV promoter (650 bases). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

127. It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

128. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

(2) Markers

129. The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once

delivered is being expressed. Preferred marker genes are the *E. Coli* lacZ gene, which encodes β -galactosidase, and green fluorescent protein.

130. In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hygromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR- cells and mouse LTK- cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

131. The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. Science 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

f) Peptides

(1) Protein variants

132. As discussed herein there are numerous variants of the ATIII protein that are known and herein contemplated. In addition, to the known functional ATIII strain variants there are derivatives of the ATIII proteins which also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence

modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of

one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

133. TABLE 1: Amino Acid Abbreviations

Amino Acid	Abbreviations
alanine	AlaA
allosoleucine	Alle
arginine	ArgR
asparagine	AsnN
aspartic acid	AspD
cysteine	CysC
glutamic acid	GluE
glutamine	GlnK
glycine	GlyG
histidine	HisH
isoleucine	IleI
leucine	LeuL
Lysine	LysK

Amino Acid	Abbreviations
phenylalanine	PheF
proline	ProP
pyroglutamic acidp	Glu
Serine	SerS
threonine	ThrT
tyrosine	TyrY
tryptophan	TrpW
Valine	ValV

TABLE 2:Amino Acid Substitutions
Original Residue Exemplary Conservative Substitutions, others are known in the art.
Alaser
Arglys, gln
Asngln; his
Aspglu
Cysser
Glnasn, lys
Gluasp
Glypro
Hisasn;gln
Ileleu; val
Leuile; val
Lysarg; gln;
MetLeu; ile
Phemet; leu; tyr
Serthr
Thrser
Trptyr
Tyrtrp; phe
Valile; leu

134. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

135. For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations
 5 such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

136. Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other
 10 labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

137. Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are
 15 frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular*
 20 *Properties*, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

138. It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. For example, SEQ ID NO:1 sets forth a
 25 particular sequence of ATIII. Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

139. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity

method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

140. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

141. It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

142. As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. For example, one of the many nucleic acid sequences that can encode the protein sequence set forth in SEQ ID NO:1 is set forth in SEQ ID NO:2. It is also understood that while no amino acid sequence indicates what particular DNA sequence encodes that protein within an organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein in the particular organism from which that protein arises is also known and herein disclosed and described.

143. It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino acids or amino acids which have a different functional substituent than the amino acids shown in Table 1 and Table 2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., *Methods in Molec. Biol.* 77:43-73 (1991), Zoller, *Current Opinion in Biotechnology*, 3:348-354 (1992); Ibba, *Biotechnology &*

Genetic Engineering Reviews 13:197-216 (1995), Cahill et al., TIBS, 14(10):400-403 (1989); Benner, TIB Tech, 12:158-163 (1994); Ibba and Hennecke, Bio/technology, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

144. Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include $\text{CH}_2\text{NH--}$, $\text{--CH}_2\text{S--}$, $\text{--CH}_2\text{--CH}_2\text{--}$, --CH=CH-- (cis and trans), $\text{--COCH}_2\text{--}$, $\text{--CH(OH)CH}_2\text{--}$, and $\text{--CHH}_2\text{SO--}$ (These and others can be found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci (1980) pp. 463-468; Hudson, D. et al., Int J Pept Prot Res 14:177-185 (1979) ($\text{--CH}_2\text{NH--}$, $\text{CH}_2\text{CH}_2\text{--}$); Spatola et al. Life Sci 38:1243-1249 (1986) ($\text{--CH H}_2\text{--S}$); Hann J. Chem. Soc Perkin Trans. I 307-314 (1982) (--CH--CH-- , cis and trans); Almquist et al. J. Med. Chem. 23:1392-1398 (1980) ($\text{--COCH}_2\text{--}$); Jennings-White et al. Tetrahedron Lett 23:2533 (1982) ($\text{--COCH}_2\text{--}$); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) ($\text{--CH(OH)CH}_2\text{--}$); Holladay et al. Tetrahedron. Lett 24:4401-4404 (1983) ($\text{--C(OH)CH}_2\text{--}$); and Hruby Life Sci 31:189-199 (1982) ($\text{--CH}_2\text{--S--}$); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is $\text{--CH}_2\text{NH--}$. It is understood that peptide analogs can have more than one atom between the bond atoms, such as b-alanine, g-aminobutyric acid, and the like.

145. Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

146. D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

g) Pharmaceutical carriers/Delivery of pharmaceutical products

147. As described above, the compositions can also be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

148. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant. As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

149. Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

150. The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem.,

2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br. J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425, (1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol., 42:2062-2065, (1991)).

5 Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)).
10 In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The
15 internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of
20 receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

(1) Pharmaceutically Acceptable Carriers

151. The compositions, including antibodies, can be used therapeutically in combination with a pharmaceutically acceptable carrier.

25 152. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of
30 the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that

certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

153. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions
5 such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

154. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice.

10 Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

155. The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally,
15 by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The disclosed antibodies can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

156. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol,
20 polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on
25 Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

157. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

30 158. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable..

159. Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

(2) Therapeutic Uses

160. Effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are effected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example, guidance in selecting appropriate doses for antibodies can be found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

h) Chips and micro arrays

161. Disclosed are chips where at least one address is the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also disclosed are chips where at least one address is the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

162. Also disclosed are chips where at least one address is a variant of the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also

disclosed are chips where at least one address is a variant of the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

i) Computer readable mediums

163. It is understood that the disclosed nucleic acids and proteins can be represented as a sequence consisting of the nucleotides of amino acids. There are a variety of ways to display these sequences, for example the nucleotide guanosine can be represented by G or g. Likewise the amino acid valine can be represented by Val or V. Those of skill in the art understand how to display and express any nucleic acid or protein sequence in any of the variety of ways that exist, each of which is considered herein disclosed. Specifically contemplated herein is the display of these sequences on computer readable mediums, such as, commercially available floppy disks, tapes, chips, hard drives, compact disks, and video disks, or other computer readable mediums. Also disclosed are the binary code representations of the disclosed sequences. Those of skill in the art understand what computer readable mediums. Thus, computer readable mediums on which the nucleic acids or protein sequences are recorded, stored, or saved.

164. Disclosed are computer readable mediums comprising the sequences and information regarding the sequences set forth herein. Also disclosed are computer readable mediums comprising the sequences and information regarding the sequences set forth herein wherein the sequences do not include SEQ ID Nos: 37, 38, 39, 40, 41, and 42.

j) Kits

165. Disclosed herein are kits that are drawn to reagents that can be used in practicing the methods disclosed herein. The kits can include any reagent or combination of reagent discussed herein or that would be understood to be required or beneficial in the practice of the disclosed methods. For example, the kits could include primers to perform the amplification reactions discussed in certain embodiments of the methods, as well as the buffers and enzymes required to use the primers as intended.

D. Methods of making the compositions

166. The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

1. Nucleic acid synthesis

167. For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic

digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System I Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al.*, *Ann. Rev. Biochem.* 53:323-356 (1984), (phosphotriester and phosphotriester methods), and Narang *et al.*, *Methods Enzymol.*, 65:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* 5:3-7 (1994).

2. Peptide synthesis

168. One method of producing the disclosed proteins is to link two or more peptides or polypeptides together by protein chemistry techniques. For example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert*-butoyloxycarbonyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) *Synthetic Peptides: A User Guide*. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) *Principles of Peptide Synthesis*. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis)).

169. Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. For example, the proteins, peptides, and polypeptides, can be produced in systems which produce fully and appropriately glycosylated versions of the ATIII. For example, advances in recombinant glycoprotein production methods, which allow more cost effective production of human glycoproteins by colonies of transgenic rabbits (www.bioprotein.com) or by yeast strains carrying human N-glycosylation system enzymes (Hamilton, S., *et al.*, (2003), *Science* 301, 1244-1246) can be used.

170. Once isolated, independent peptides or polypeptides may be linked, if needed, to form a peptide or fragment thereof via similar peptide condensation reactions. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. *Synthesis of Proteins by Native Chemical Ligation*. Science, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) *FEBS Lett.* 307:97-101; Clark-Lewis I et al., *J.Biol.Chem.*, 269:16075 (1994); Clark-Lewis I et al., *Biochemistry*, 30:3128 (1991); Rajarathnam K et al., *Biochemistry* 33:6623-30 (1994)).

171. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., *Techniques in Protein Chemistry IV*. Academic Press, New York, pp. 257-267 (1992)).

E. Examples

172. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1 – Shear rate dependent partitioning of plasma derived antithrombin III isoforms and high-heparin-affinity recombinant ATIII between circulating blood and vascular surfaces

a) Results

173. Disclosed herein the production of two forms of antithrombin III with different heparin/HSPG affinities has been evolutionarily conserved because it promotes favorable partitioning of ATIII between the blood and vascular surfaces in contact with the blood under physiological conditions of flow. This is supported by experiments disclosed herein in which human plasma derived antithrombin isoforms and recombinant ATIII were pumped through heparin-coated CBAS tubing in an effort to model of blood flowing through the HSPG lined circulatory system.

174. As illustrated in Fig. 1, regions where wall-shear-rates approximated those in the arterial and venous compartments were generated by compressing one half of the length of the CBAS tubing and allowing the other half to remain uncompressed. For example, at a volumetric flow rate of 1.4 ml/min, solutions flowing over luminal surfaces in the compressed and non-compressed sections of the tubing were exposed to wall-shear-rates of $\sim 2000 \text{ sec}^{-1}$ and $\sim 9 \text{ sec}^{-1}$, respectively. Samples containing various mixtures of plasma-derived alpha and beta ATIII isoforms and DES.N135A recombinant ATIII, a prototypical super-beta-ATIII, were injected into the circuit and exposed to it for various periods of time, at various flow rates. Then, after washing with normal saline, the tubing was cut into segments corresponding to regions with different shear rates. Surface-bound ATIII was eluted from the tubing pieces with SDS - bmercaptoethanol gel buffer, and ATIII isoform composition determined by SDS-PAGE.

175. As discussed herein, the synthetic ratio of the antithrombin isoforms is $50\alpha:50\beta$, but they circulate in blood at a ratio of $\sim 90\alpha:10\beta$. A solution composed of $50\alpha:50\beta$ was pumped through the Fig. 1 circuit for 4 hours. The total amount of ATIII in the recirculated sample was double the ATIII binding capacity of the CBAS tubing. At the end of the recirculation period, the ATIII solution was collected and the circuit was washed with normal saline. The CBAS tubing from low and high wall-shear-rate sections of the circuit was cut into 2 cm segments, and bound ATIII eluted with SDS- β mc. The isoform contents of pre- and postcirculation solutions and the CBAS tubing eluates were determined by 12% polyacrylamide gel electrophoresis as illustrated in Fig. 2.

176. Fig. 2 shows that after 4 hours of flow in the *in vitro* model of the circulatory system, the $\alpha:\beta$ ratio of the "blood" shifted from $50\alpha:50\beta$ (lane a) to $\sim 90\alpha:10\beta$ (lane b), and that the heparincoated CBAS tubing representing the vascular endothelium bound mainly β -ATIII. The quantity of ATIII bound per unit of luminal surface area was greatest in low shear rate (venous) segments of the circuit, and reduced in the high shear rate (arterial) sections.

177. Fig. 3 shows differential binding of ATIII isoforms to the heparin-coated CBAS tubing as a function of exposure time under zero-flow, low or high wall-shear-rate conditions. In this study, circuits were loaded with a sample that had an isoform ratio of $>90\alpha:<10\beta$, which is similar to plasma. The "pre" lane on the left side of Fig. 3 shows that β -antithrombin is barely detectable in this sample. In contrast, significant amounts of β -ATIII are observed on the tubing surface following exposure to the $>90\alpha:<10\beta$ sample under various time, flow, and geometry conditions.

178. Under static, zero-shear conditions (A), the surface-bound $\alpha:\beta$ ratio initially resembled that of the "pre" sample. However, with increased exposure time, the total amount of surface bound ATIII and its $\beta:\alpha$ ratio increased, consistent with the greater k_{on} and reduced k_{off} that have been measured for the beta isoform under solution equilibrium binding conditions (Turk, B., et al., (1997), *Biochemistry* 36, 6682-6691) and progressive displacement of the α -isoform from heparin receptors on the wall.

179. The pattern of isoform and total antithrombin binding to the wall under low wall-shear-rate conditions (B) was qualitatively similar to the pattern obtained under static conditions. Quantitatively, however, more antithrombin bound to the surface under low wall-shear-rate conditions due to recirculation-associated exposure of the heparin receptors to more molecules of ATIII.

180. The pattern of ATIII isoform surface binding in the high shear rate section of the circuit (C) is quite different from the patterns obtained under zero-flow and low shear rate conditions. Under high shear rate conditions, minimal ATIII- α bound to the surface, whereas β -ATIII binding increased with time, resulting in a surface-bound ratio of $\sim 80\beta:20\alpha$ after 2 hours. Apparently, a large differential in isoform binding affinities develops at high wall-shear-rates. This leads to reduced surface accumulation of α -ATIII, and to stable and cumulative binding of β -ATIII.

181. Finally, different patterns of isoform binding were observed in the non-compressed vs. compressed sections of the circuit under zero-flow conditions (see panels A vs. D of Fig. 3, corresponding to segments 2 vs. 6 of Fig. 1). These differences suggest that vessel diameters (which vary greatly through out the circulatory tree and lead to large variations in surface area to blood volume and diffusion distance values) also affect how antithrombin isoforms partition between the blood and the vessel wall.

182. Results from the Fig. 2 and 3 experiments suggest that the difference in α -ATIII and β -ATIII binding affinities for heparin is magnified under flowing conditions, and that this leads to preferential binding of β -ATIII on vascular surfaces and α -ATIII relegation to the blood, as is observed physiologically. Moreover, the Fig. 3 experiment demonstrates that despite its low initial concentration in the circulating sample, β -ATIII is still efficiently sequestered from the fluid phase to target and bind the heparin-coated surface. This observation indicates that "super" β -antithrombin molecules with further increases in their heparin affinity would offer additional dosing advantages with respect to loading vascular and heparin-coated medical device surfaces with antithrombotic and anti-inflammatory ATIII.

183. To investigate this possibility, a solution containing plasma derived α -ATIII, plasma derived β -ATIII and recombinant DES.N135A ATIII was circulated through the *in vitro* circulatory system model. The measured solution equilibrium binding K_s of plasma α , plasma β and recombinant DES.N135A for high affinity heparin at pH 7.4 and 0.3 I are respectively 300 nM, 54 nM and 6 nM (Turk, B., et al., (1997), *Biochemistry* 36, 6682-6691). This means that under zero-flow conditions, plasma β -ATIII exhibits a 6-fold higher affinity for heparin than does plasma α -ATIII, and that recombinant DES.N135A ATIII has a 50- fold higher affinity for heparin than does plasma α -ATIII, and a 9-fold higher affinity for heparin than does plasma β -ATIII.

184. The left lanes of Fig. 4 show a sample initially containing α , β and DES.N135A antithrombins in an ~40:40:20 ratio before (lane a) and after (lanes b and c) 15 minutes of recirculation. The fluid phase was selectively depleted of the DES.N135A species that has the highest affinity for heparin due to its capture on surfaces of the proximal low wall-shear-rate and high wall-shear-rate sections of the circuit. The species with the next highest heparin affinity, plasma derived β -antithrombin, bound to surfaces in low and high wall-shear-rate sections of the circuit. Finally, the lowest affinity ATIII- α isoform bound only to surfaces in low shear rate segments of the circuit. Collectively, these binding patterns indicate that as the wall-shear-rate increases, corresponding increases in ATIII heparin/HSPG binding affinities are required to mediate stable binding to the surface. Conversely, high wall-shear-rates are non-permissive for binding of lower affinity antithrombins (e.g. α -ATIII) to the wall.

185. Additionally, binding of DES.N135A and β -ATIII is inferred to occur very rapidly, based on the greater presence of these species on proximal vs. distal low wall-shear-rate regions of the circuit. The increased binding of α -ATIII observed in distal vs. proximal low

wall-shear-rate regions of the circuit probably results from increased receptor availability due reduced DES.N135A and β -ATIII binding in this region.

186. The Fig. 2-4 experiments indicate that β -antithrombins with enhanced affinities for heparin/HSPG are useful at low doses for augmenting ATIII-mediated antithrombotic and anti-inflammatory activities on the vessel surface, especially in regions of the circulation having high wall-shear-rates.

187. Equilibrium binding measurements of antithrombin affinities for heparin conducted with both the ligand and receptor in solution have been used to establish the relative affinities of plasma ATIII isoforms and recombinant antithrombins for heparin. However, the disclosed studies with antithrombin isoforms flowing through a CBAS heparin-coated circuit indicate that several additional factors must be considered in order to properly describe the physiologically relevant interaction of antithrombin III binding to vascular wall heparan sulfate proteoglycans. Primary among these considerations are that (1) the HSPG receptors are immobilized on the vessel surface, rather than free in solution like pharmaceutical heparin, and that (2) due to variations in flow rates and vessel geometry in different parts of the circulatory tree, the binding reaction actually occurs under a wide range of wall-shear-rates.

188. In a semi-quantitative way, the 3 mm CBAS tubing studies show that high wall-shear-rates are less permissive for ATIII- α isoform binding to surface-bound heparin, and that with increasing shear rates, β -ATIII is preferentially bound. These studies also reveal that due to its higher affinity, the β -isoform can still effectively target the surface when its concentration in the circulating fluid phase is very low compared to that of the α -isoform that predominates in circulating blood. This indicates that at very low doses "super" β -antithrombins with enhanced heparin affinity can effectively target ATIII antithrombotic and anti-inflammatory activity to the vessel wall.

2. Example 2

a) BACKGROUND

189. Antithrombin III (ATIII) is a plasma proteinase inhibitor that inactivates coagulation enzymes such as thrombin and factor Xa (FXa). ATIII binding to vascular surface heparan sulfate proteoglycan (HSPG) receptors and pharmaceutical heparin allosterically accelerates its thrombin and FXa inhibition rates by a factor of ~1000-fold. There are two ATIII isoforms, α -ATIII and β -ATIII, which have 4 and 3 N-linked glycans, respectively. The β -isoform has higher affinity for the ATIII-binding pentasaccharide sequence of HSPG/heparin

than the α isoform. Isoform production is evolutionarily conserved. This suggests that it is advantageous to carry both isoforms, and that α and β have distinct and critical functions.

190. The ATIII isoforms are synthesized in a $\sim 50\alpha:50\beta$ ratio. The α isoform predominates in circulating blood. The ATIII isoform ratio of plasma is $\sim 90\alpha:10\beta$. The β isoform interacts preferentially with the vascular endothelium. β -ATIII clears from the blood more rapidly than α -ATIII, and is present at increased concentrations on rabbit aorta intima/media. HSPG bound ATIII contributes substantially to anticoagulant and antithrombotic properties of the endothelium.

191. Disclosed herein rheological factors influence ATIII isoform interactions with vascular surfaces and heparin-coated medical devices. The wide range of wall shear rates encountered in different regions of the vascular tree can contribute to differential partitioning of ATIII isoforms between the circulating blood and HSPG-containing vascular surfaces and heparin-coated medical devices. Figure 1 shows a diagram of an in vitro system which can be used to show this. The circuit was constructed from uncoated and heparin-coated (Carmeda BioActive Surface, CBAS) polyvinyl chloride (PVC) tubing. Half of the CBAS tubing length was compressed in order to generate sections with high (segments #4-9) and low (segments #1-3 and #10-11) wall shear rates. During recirculation at a volumetric flow rate of 1.4 ml/min, wall shear rates were respectively 9 sec^{-1} and $2,000 \text{ sec}^{-1}$ in the uncompressed and compressed sections of the heparin-coated tubing. Samples containing 1.5uM total ATIII at different $\alpha:\beta$ ratios were injected into the loop and recirculated for various times. For static experiments, a no-flow rate of 0 ml/min was maintained for the indicated periods after sample injection. At the end of the recirculation/exposure period, the fluid phase containing unbound ATIII was collected, and the circuit washed with buffered 0.15 M NaCl. The heparin-coated tubing was cut into 2-cm segments #1 - #12, as illustrated above. Surface-bound antithrombin was eluted from each segment with SDS- β me gel buffer, and the isoform content determined by 12% PAGE.

192. Fluid phase results after 4 hours exposure to the circuit under static, no-flow conditions, the $\alpha:\beta$ ratio of the fluid phase (lanes b and c) remained similar to that of the injected sample (lanes a and d). Surface-bound ATIII result are shown and the isoform ratio of ATIII that bound the heparin coated tubing (CBAS segments #2 - #12) under static conditions exhibited a slight increase in β -ATIII content relative to α -ATIII content.

In Figure 2 the fluid phase results are shown after 4 hours exposure to the circuit under flowing conditions, the $\alpha:\beta$ ratio of the fluid phase changed from $\sim 50\alpha:50\beta$ in the

original sample (lanes a and b) to $\sim 90\alpha : 10\beta$ in the post circulation sample (lane c). Also shown are the surface-bound ATIII results which show the isoform ratio of surface-bound ATIII eluted from CBAS segments #1 - #12 exhibited increased content of the β -ATIII isoform relative to α -ATIII isoform. Low shear rate segments (#1, #2, #10 and #12) appeared to bind more ATIII per unit of luminal surface area than high shear rate segments (#4, #6 and #8) of the circuit.

194. Figure 2 shows that the conditions for experiments #1 and #2 were identical except for the use of static, no-flow vs. flowing conditions to introduce a wall shear rate variable. The binding of both isoforms to the heparin-coated surface decreased as the wall shear rate increased, however, α -ATIII binding was reduced to a greater extent than β -ATIII binding. After prolonged exposure to a sample of initial $50\alpha : 50\beta$ composition, low and high wall shear rate sections of the circuit both bound mainly β -ATIII, and the isoform ratio in fluid phase became $90\alpha : 10\beta$. Thus, the *in vitro* flow model partitioned the isoforms in a physiologically accurate manner. The surface density of ATIII bound to high shear rate (arterial-like) segments of the circuit was less than that in the low shear rate (venous-like) segments. These experiments indicate that flow is an important factor in partitioning of antithrombin III isoforms between the blood and HSPG-lined vascular surfaces.

195. Figure 3 shows surface-bound ATIII results under Static and No-Flow conditions (experiments A and D). The initial isoform content of the injected sample was $90\alpha : 10\beta$. The β -isoform was only weakly visible on PAGE of this sample (a lanes). For the earliest 3 min. time point of the static experiment, the isoform ratio of surface-bound ATIII from the uncompressed tubing segment #2 (A) resembled that of the input sample. However, as exposure time increased, less α -ATIII and more β -ATIII were recovered. Therefore, under static conditions in the low (1.33) surface area-to-volume, low receptor-to-ligand segment of the circuit, ATIII isoforms initially bound the CBAS surface according to the law of mass action. With increased exposure time, α was displaced and β -ATIII accumulated on the surface, consistent with β 's higher heparin affinity. Surface bound ATIII from compressed tubing segment #6 (D) exhibited a $\sim 50\alpha : 50\beta$ isoform ratio at all time points of the static, no-flow experiment. In this case, the high (20.4) surface area-to-volume ratio indicates that a reduced number of ligand molecules are available to the same number of receptors. Consequently, β -ATIII saturation of the surface-bound ATIII profile appears to occur earlier in the time course, and significant α -ATIII displacement does not occur due to the low β -ATIII content in the fluid

phase of the compressed segment. In Figure 3 surface-bound ATIII results under Flowing conditions (experiments B and C) are shown. In presence of flow at a low wall shear rate (9 sec^{-1}) (B), the surface-bound ATIII isoform composition of CBAS segment #2 initially resembled that of the injected sample and was low in β -ATIII content. However, with longer exposure times, both the absolute amount of bound ATIII and its β -ATIII content increased progressively, reaching $\sim 50\alpha : 50\beta$ at 2 hours. In contrast, initial and subsequent isoform profiles of surface-bound ATIII recovered from the segment #6 high shear rate ($2,000 \text{ sec}^{-1}$) section of the circuit (D) differed greatly from the composition of the injected sample. Whereas the isoform ratio of the injected sample was $\sim 90\alpha : 10\beta$ (lane a), the composition of surface bound ATIII was $\sim 40\alpha : 60\beta$ at 3 minutes, and $\sim 10\alpha : 90\beta$ at 2 hours.

196. Kinetic studies of **a** and **b** ATIII binding to a heparin-coated surface suggest that distinct, shear rate-dependent mechanisms influence the isoform composition of surface-bound ATIII in low vs. high shear rate regions of the vasculature. No-flow conditions and low wall shear rates are permissive for the binding of both ATIII isoforms, which are initially recovered from these surfaces in accordance with their proportions in the fluid phase. Then, **b**-ATIII progressively displaces **a**-ATIII, due to presumably faster k_{on} and slower k_{off} values associated with **b**-ATIII's enhanced affinity for heparin. At higher wall shear rates, conditions are permissive for the binding of **b**-ATIII, but become less permissive for **a**-ATIII binding. **b**-ATIII content on these high shear rate surfaces is therefore initially high and exhibits further increases with time.

197. This study demonstrates that rheological factors influence ATIII isoform interactions with heparin-coated surfaces. The work further indicates that the high **a**-ATIII content of the blood and the reciprocally high **b**-ATIII content of vascular surfaces result from shear rate-dependent partitioning of the isoforms between the blood and vascular surfaces. It was also demonstrated that over a broad range of physiologically and pathologically relevant shear rates, **b**-ATIII efficiently bound a heparin-coated surface, despite its initially trace concentration in the fluid phase. This finding suggests that low dose **b**-ATIII and **b**-ATIII derivatives are particularly well suited for augmenting the anticoagulant and antithrombotic properties of vascular surfaces and heparin-coated medical devices.

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G. Sequences

1. SEQ ID NO:1 Protein sequence Human ATIII Accession no. AH004913.

MYSNVIGTVTSGRKVYLLSLLLIGFWDCVTCHGSPVDICTAKP

RDIPNPMCIYRSPEKKATEDEGSEQIPEATNRRVWELSKANSRFAITTFYQHLADSK
 NNDNINFLSPLSISTAFAMTKLGACNDTLQQLMEVFKFDITSEKTSQDIHFHFAKLC
 RLYRKANKSSKSLVSANRLFGDKSLTFNETYQDISELVYGAQLPLDFKENAQESRAAI
 NKWVSNKTEGRITDVIPSEAINELTVLVLVNTIYFKGLWKSFKSPENTRKELFYKADG
 ESCSASMMYQEGKFRYRRVAEGTQVLELFPKGGDDITMVLILPKPEKSLAKVEKELTPE
 VLQEWLEDEEEMMLVHMPRFRIEDGFSLEQLQDMGLVDLFSPEKSKPLGPIVAEGRD
 DLYVSDAFHKAFLVNEEGSEAAASTA VVIAGRSLNPNRVTFKANRPFLVFIREFVPLN
 TIIFMGRVANPCVK

2. SEQ ID NO:2 cDNA sequence Human ATIII Accession no. 103102.

1 caccagcacc atctctctcca attcatccag ctactctgcc catgaagata atagttttca
 61 ggccgattgc ctcatgacac actatctcca ctctgccagc cctgtggaag attagcgccc
 121 atgtattcca atgtgatagg aactgtaaac tctggaaaaa ggaagggtta tcttttgtcc
 181 ttgctgctca ttggcttctg ggactgcgtg acctgtcacg ggagccctgt ggacatctgc
 241 acagccaagc cgcgggacat tcccatgaat cccatgtgca ttaccgctc cccggagaag
 301 aaggcaactg aggatgaggg ctacagaacg aagatcccg aggccaccaa ccggcgctgc
 361 tgggaactgt ccaaggccaa tcccgcttt gctaccactt tctatcagca cctggcagat
 421 tccaagaatg acaatgataa catcttctgt taccctctga gtatctccac ggcctttgtg
 481 atgaccaagc tgggtgcctg taatgacacc ctccagcaac tgatggaggt atttaagttt
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 781 tgggtgtcca ataaagaccga aggcgaatc atttacttca agggcgctgt gaagtcaag
 841 gagctcaact ttctgggtgt ggttaacacc ctctatcgcc cgtggctga aggcaccag
 901 ttcagccctg agaacacaag gaaggaaactg ttctacaagg ctgatggaga gtcgtgttca
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 1201 ttcagtttga aggacagcgt cgaagacatg ggctttgtcg atctgttcaag cctgaaagg
 1261 tccaaactcc caggtattgt tgcagaagcg cgagatgacc tctatgtctc agatgacttc
 1321 cataagagcat tctttgaggt aaatgaagaa ggcagtgaa gacgtgtgaag taccgctgtt
 1381 gtgattgctg gccgttcgct aaaccccaac aggttgactt tcaaggccaa caggcccttc
 1441 ctggttttta taagagaagt tcccttgaac actatattct tcatggcgag agtagccaac

1501 ccttggtta agtaaaatgt tcttattctt tgcacctctt cctatttttg gtttgtgaac
1561 agaagtaaaa ataaatacaa actacttcca tctcacatt

V. CLAIMS

What is claimed is:

1. A method of decreasing coagulation or thrombosis in a system, comprising administering an ATIII molecule, wherein the ATIII molecule has an increased
5 affinity for heparin or heparin sulfate proteoglycans bound to a solid surface, and wherein the majority of the ATIII binds the heparin or heparin sulfate proteoglycans under high wall shear rate conditions.
2. A method of inhibiting coagulation under low and high shear rate conditions comprising administering an ATIII molecule, wherein the majority of the ATIII
10 molecule binds heparin or heparin sulfate proteoglycans under low and high shear rate conditions. Couch in terms of assay
3. A method of inhibiting coagulation during a cardiovascular procedure on a subject comprising administering high affinity ATIII molecules to the subject, wherein the majority of the ATIII molecules bind heparin or heparin sulfate
15 proteoglycans under low and high shear rate conditions.
4. A method of preconditioning a heparin or heparin sulfate polyglycan coated material, comprising incubating the material with a solution comprising ATIII molecules, such that a majority of the ATIII molecules bind to the heparin or heparin sulfate proteoglycans under low and high shear rate conditions.
- 20 5. The method of claim 1, further comprising a determination that high wall shear rates on the ATIII will occur.
6. The method of claim 1, wherein the system comprises a stent.
7. The method of claim 1, wherein the ATIII comprises beta ATIII.
8. The method of claim 1, wherein the solid surface comprises a metal.
- 25 9. The method of claim 1, wherein the shear rate conditions comprise shear rates of at least 1000 sec^{-1} .
10. The method of claim 1, wherein the shear rate conditions comprise shear rates of at least 1500 sec^{-1} .
11. The method of claim 1, wherein the shear rate conditions comprise shear rates of
30 at least 2000 sec^{-1} .

12. The method of claim 1, wherein the shear rate conditions comprise shear rates of at least 2500 sec^{-1} .

13. The method of claim 1, wherein the shear rate conditions comprise shear rates of at least 3000 sec^{-1} .

5 14. The method of claim 1, wherein the dissociation constant of ATIII for heparin is at least 54 nM.

15. The method of claim 1, wherein the dissociation constant of ATIII for heparin is at least 6 nM.

16.

10

VI. ABSTRACT OF THE DISCLOSURE

222. Disclosed are compositions and methods related to binding of ATIII under low and high shear rate conditions.

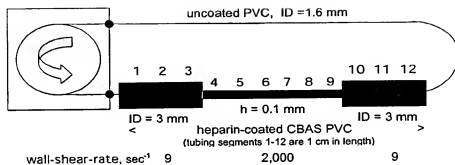


FIG. 1

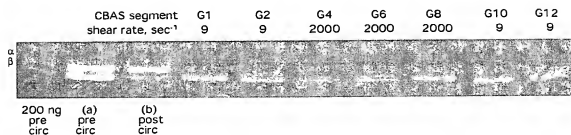


FIG. 2

Surface-bound ATIII												
wall-shear-rate, sec ⁻¹ exposure time, min surface area/vol, mm ² /mm ³	A			B			C			D		
	0			9			2000			0		
	3	15	120	3	15	120	3	15	120	3	15	120
	1.33	1.33	1.33	1.33	1.33	1.33	20.4	20.4	20.4	20.4	20.4	20.4
pre												
circ												
1:1 α/β std												

FIG. 3

CBAS segment				B2	B3	B5	B7	B11	B12
shear rate, sec ⁻¹				9	9	2000	2000	9	9
DES.									
N135A									
10 ul	10 ul	10 ul							
pre	post	post							
circ	circ	circ							
(a)	(b)	(c)							
									400 ng
									pre
									circ

FIG. 4